Supporting Information

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SI Materials and Methods

Quantification of *pyebl* and *pyama1* Transcripts. To quantify and compare *pyebl* transcription levels between 17X and 17XL, real-time PCR was carried out under the following conditions. Five BALB/c mice were infected with *P. yoelii* i.p., and parasites were collected on day 5 by cardiac puncture. Total RNA was isolated from each mouse independently, and cDNA was generated from the schizont-enriched fraction. Real-time PCR was carried out using the QuantiTect SYBR Green PCR Kit (Qiagen) with 5′-TGAATCTTTCCAATCTTTCCC-3′ and 5′-CCATGTCTCTCCGTTTTCAATG-3′ primers for *pyebl* or with 5′-GAAAAGGTGCATGGTTCTGG-3′ and 5′-GAAAAGGTGCATGGTTCTGG-3′ primers for *pyama1* using the LightCycler system (F. Hoffmann-La Roche). Copy numbers of *pyebl* and *pyama1* were determined using a standard curve generated by serially diluted plasmids containing the target insert sequence.

SDS-PAGE and Western Blot Analysis. Parasite-infected blood was passed through a CF11 cellulose column to remove leukocytes, and a schizont-enriched fraction was collected by differential centrifugation on a 50% Percoll solution. Schizont proteins were first extracted by repeated freeze–thaw cycles in PBS containing protease inhibitors [PI; 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 100 μM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride] and 1 mM EDTA, followed by extraction of the insoluble pellet in 1% Triton X-100 in PBS-PI. Triton X-100 soluble proteins, containing *Py*EBL and *Py*AMA1, were incubated with 6% β-mercaptoethanol at 100 °C for 3 min and subjected to electrophoresis on a 5%–20% polyacrylamide gel (ATTO). Proteins were then transferred to a Immuno-Blot PVDF membrane (Bio-Rad), immunostained with mouse serum or monoclonal antibodies followed by HRP-conjugated goat anti-(mouse IgG Fc) antibody (The Jackson Laboratory), and visualized with Immobilon Western Chemiluminescent HRP substrate (Millipore). Images were captured on RX-U film.

Live Staining of *P. yoelii* Free Merozoites. Parasitized erythrocytes collected from mice were suspended in RPMI 1640 medium containing 20% FCS (complete medium) and incubated at 24 °C for 13 h for schizont maturation. Schizonts were then enriched by differential centrifugation on 50% HistoDenz (Sigma) in PBS at 1,130 × *g* for 12 min. Collected parasites were passed through a 29-gauge syringe needle 5 times, suspended in 500 μL of RPMI 1640 complete medium, and then incubated at 37 °C for 2 min. Parasites were washed, incubated in RPMI 1640 complete medium containing rabbit anti-*Py*EBL R3–5 and mouse anti-*Py*MSP1–19 sera at room temperature for 30 min, washed again, and smeared on glass slides. Air-dried smears were fixed in ice-cold acetone and incubated with Alexa-488–conjugated goat antirabbit IgG antibody (Molecular Probes) and Alexa-546–conjugated goat antimouse IgG antibody. Parasite nuclei were stained with DAPI. Images were captured as described above.

Knock-Out Strategy of the *pyebl* Gene Locus. To knock out the *pyebl* gene locus, a DNA fragment from the 5′ side of *pyebl* was PCR-amplified from gDNA of the *P. yoelii* 17XL line with primers 5′-ACCAAATGCATAGAGTTTTA-3′ and 5′-AAATTGATTCTTCTACTTGGTATG-3′. PCR products were then ligated into SmaI site of pR6Cyt-B12, yielding pR6Cyt + 5U-B12, which was processed in a similar manner as described above to yield knock-out construct pYEBL-KO

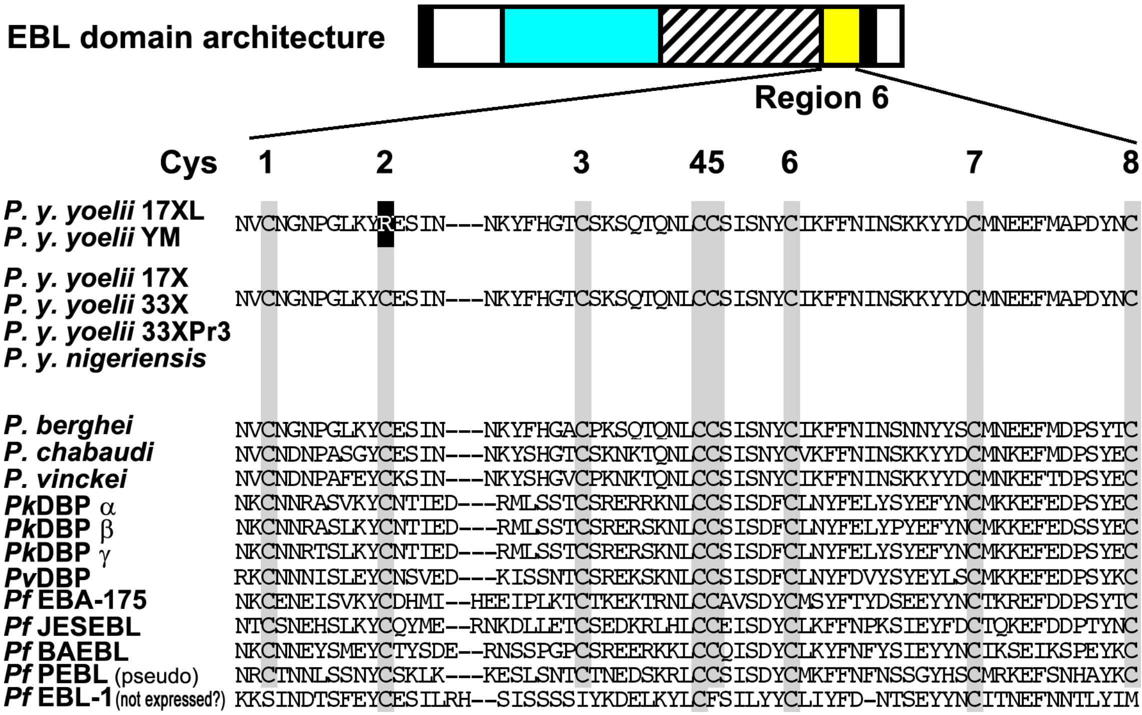

Fig. S1. Alignment of the deduced amino acid sequence of *Plasmodium* EBL region 6. Cys residues are highlighted and numbered. *P.y.*, *Plasmodium yoelii*; *Pk*DBPα, -β, and -γ (M90466, M90694, and M90695, respectively), *Plasmodium knowlesi* Duffy binding proteins; *Pv*DBP (DQ156512), *Plasmodium vivax* DBP; *Pf*EBA-175 (M93397), *Pf*JESEBL (AB080796), *Pf*BAEBL (AF332918), *Pf*PEBL (DQ100425), and *Pf*EBL1 (AF131999), *Plasmodium falciparum* EBLs. Nucleotide sequences for *P. y. yoelii* EBL 17XL line (AB430781), YM line (AB430783), 17X line (AB430782), 33X line (AB430784), and 33XPr3 line (AB430785), *P. y. nigeriensis* EBL (AB430786), *P. berghei* EBL (ANKA line, AB430787), *P. chabaudi* EBL (AB430788), and *P. vinckei* EBL (AB430789) are deposited at the DNA database in Japan. The number and location of Cys residues in region 6 are conserved among 17 EBL proteins except *P. yoelii* 17XL and YM lines and *Pf*EBL-1. The transcription of *Pf*EBL-1 has been shown to be low, the transcription pattern throughout the asexual life cycle was distinct from other members, and protein expression has not been observed to date. *P. yoelii* 33X, 33XPr3, and YM lines are kind gifts from D. Walliker. Peliminary sequence data for *P. berghei*, *P. chabaudi*, and *P. vinckei* were obtained from The Institute for Genomic Research. Nucleotide sequence data reported in this article are available in the GenBank/European Molecular Biology Laboratory/DNA Data Base in Japan databases under the accession numbers AB430781–AB430789.

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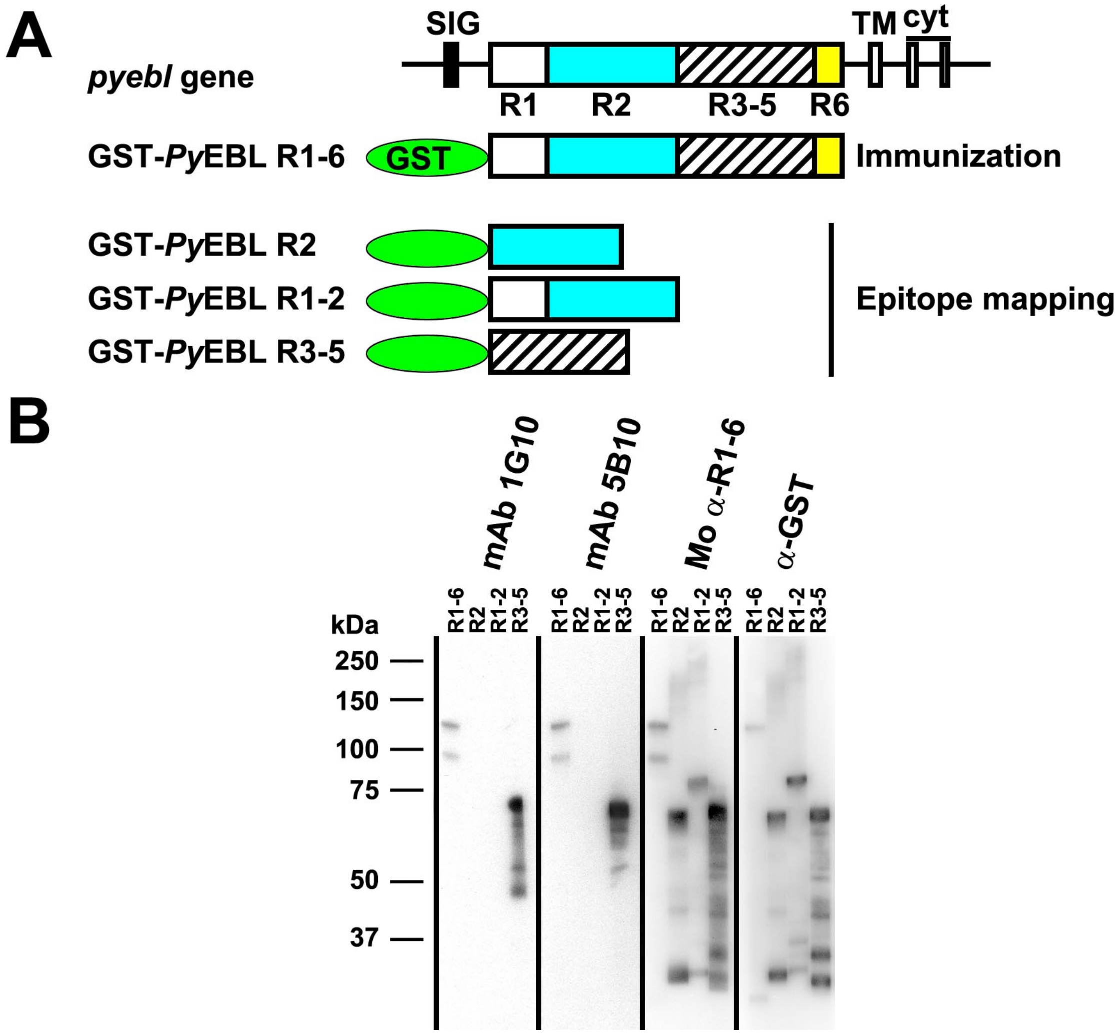

Fig. S2. Recombinant EBL proteins and anti-*Py*EBL antibodies. (*A*) Schematic representation of a panel of GST-fused *Py*EBL recombinant proteins used for immunization and/or epitope mapping of antibodies. SIG, predicted signal peptide sequence; TM, transmembrane region; cyt, cytoplasmic region; R1, R2, R3–5, and R6, *Py*EBL regions 1 to 6. (*B*) Epitope mapping of antibodies. Western blot analysis showed that mAbs 1G10 and 5B10 reacted with GST-*Py*EBLR1–6 and GST-*Py*EBLR3–5, indicating that the epitope was located in *Py*EBL region R-5. Mouse anti-*Py*EBLR1–6 serum (Mo α-R1–6) reacted with all recombinant *Py*EBL proteins.

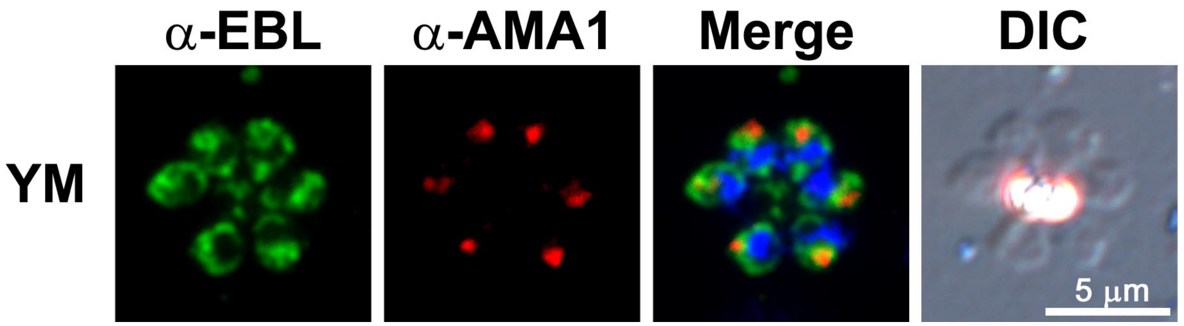

Fig. S3. Subcellular localization of EBL in *Plasmodium yoelii* YM line. *P. yoelii* YM line schizonts were incubated with mAb 5B10 (α-EBL; FITC, green), rabbit anti-AMA1 serum (α-AMA1; Alexa-546, red), and DAPI (blue) for nuclear staining. DIC images are shown in the right-hand panel. EBL did not colocalize with AMA1 in the YM line, similar to the observation in 17XL line.

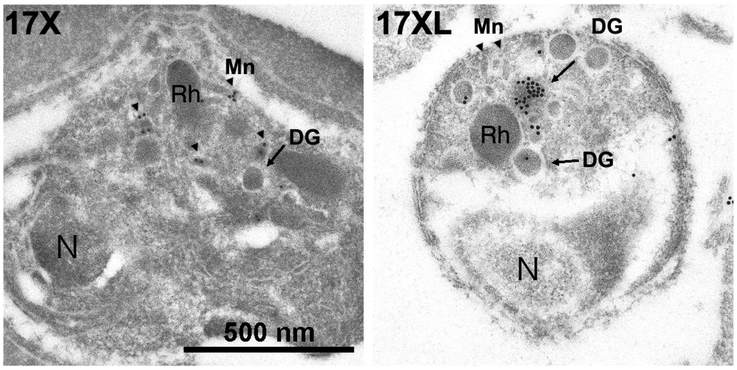

Fig. S4. Further immunoelectron microscopy images. *Py*EBL was detected in the micronemes (arrowheads) in the 17X line; however, it was detected in the dense granules (arrows) in the 17XL line. N, nucleus; Mn, micronemes; DG, dense granules; Rh, rhoptries.

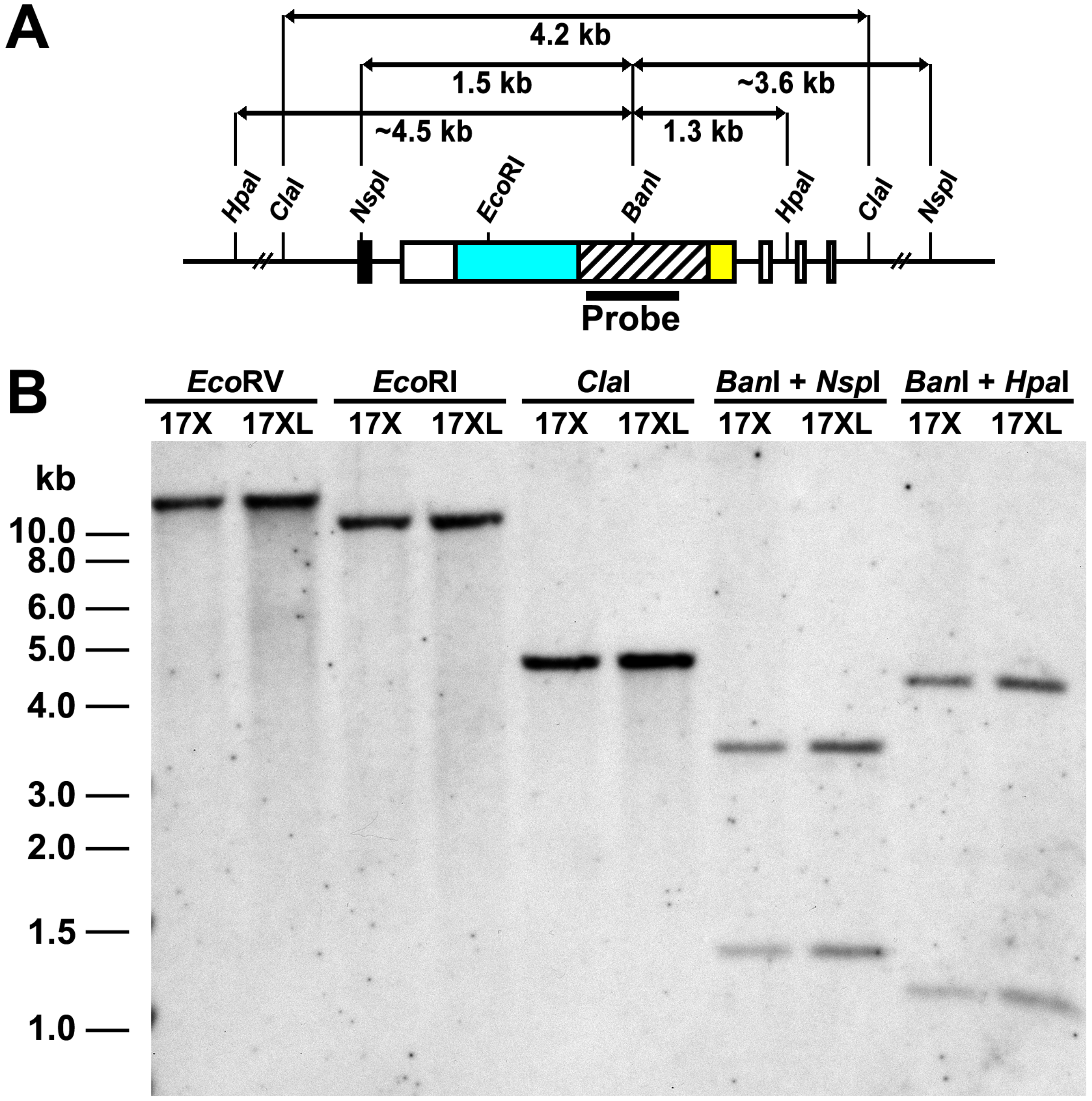

Fig. S5. *pyebl* is a single-copy gene. (*A*) Schematic of the *py*ebl gene locus (not to scale). Restriction sites and the expected size of the DNA fragment by enzyme digestion are shown. ''Probe'' indicates the probe region used in Southern blot analysis. (*B*) Southern blot analysis of the *pyebl* gene locus in the 17X and 17XL lines. Hybridization of *pyebl* probe detected only a single band from EcoRI- or EcoRV-digested DNA and only 2 bands from BanI/HpaI- or BanI/*Nsp*I-digested DNA. If the *P. yoelii* genome possesses more than a single copy of *pyebl*, additional bands are likely to be observed. Taken together with the fact that only a single copy of the *pyebl* gene can be found in the *P. yoelii* (17X line) genome database (5-fold coverage), this result strongly suggests that *pyebl* is a single-copy gene in both the 17X and 17XL lines.

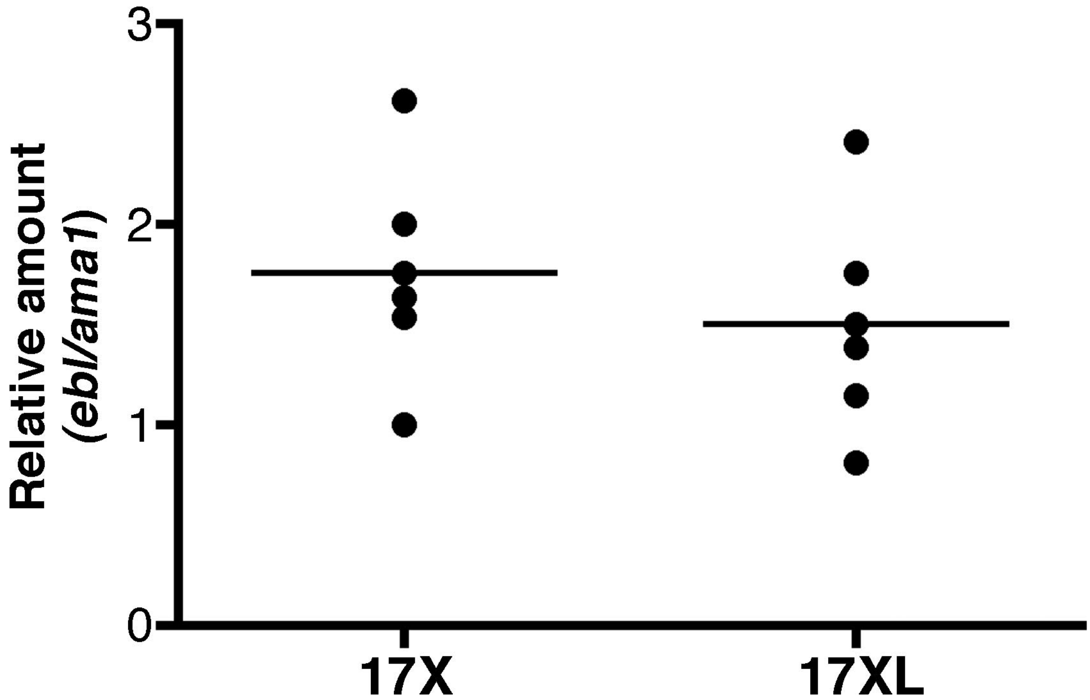

Fig. S6. Quantitative comparison of *Py*EBL transcription. No significant difference between 17X and 17XL in the relative transcript number of *pyebl* against *ama1* quantified by real-time RT-PCR ($n = 5$). Horizontal lines indicate median values.

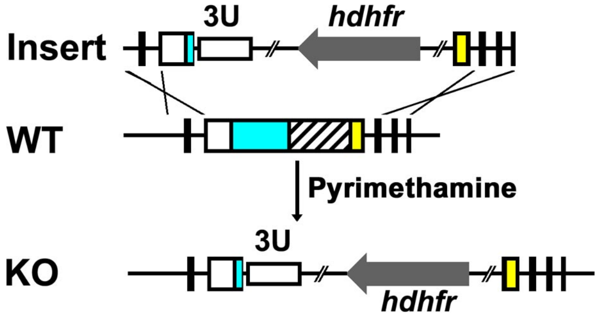

Fig. S7. Knock-out strategy of the *pyebl* gene locus. Schematic of the WT and knock-out (KO) *pyebl* gene loci. The knock-out cassette (Insert) would be inserted into the *pyebl* gene locus by double-crossover recombination and disrupts gene function. A total of 4 attempts to disrupt the *pyebl* gene locus were performed in 17X and 17XL lines (twice for each), without success. As a control, replacement plasmids, pYEBL-R6Cyt+R3Cyt(X) and pYEBL-R6Cyt+R3Cyt(XL), were simultaneously transfected, and genomic integration of these plasmids was always obtained.

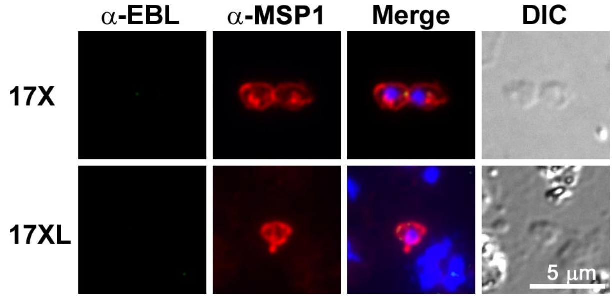

Fig. S8. *Py*EBL was not expressed on the released merozoite surface. Live merozoites of *P. yoelii* 17X and 17XL lines were incubated with rabbit anti-*Py*EBL R3–5 serum (α-EBL) and mouse anti-*Py*MSP1–19 serum (α-MSP1), followed by secondary antibodies conjugated with fluorescent Alexa dyes and DAPI for nuclear staining. DIC images are shown in the right-hand column. MSP1 was detected on the merozoite surface, but EBL was not. Anti-*Py*MSP1–19 serum was produced by N. Kangwanrangsan, Ehime University.